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Improvement on Preparation of RNA from Differentiating Xylem Tissue of *Eucalyptus camaldulensis* L.

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Abstract——The CTAB method for RNA isolation in differentiating xylem has improved in the treatment of tissues with methanol containing DTT and the addition of vanadyl ribonucleosides in to the extraction buffer. Vanadyl ribonucleosides were supposed to inhibit the binding of polyphenols to RNA also, not only inhibited RNase activity. The yield of RNA was more than 270 μg/g of tissue in average, and poly(A)$^+$ RNA was subsequently purified by using an oligo-dT cellulose column with about 0.7% recovery of total RNA.

Keywords : Eucalyptus, RNA, xylem

1. Introduction

To study the genes which are expressed in a certain tissue, it is effective for achievement to isolate RNA from the objective tissue. When the phenomenon and substances involved in wood and wood formation are studied, it is important to target on differentiating xylem. Most of wood compositions are produced in this tissue. Thus the technique providing RNA in high quality and enough yield from this tissue is required. RNA has already been isolated from differentiating xylem of several tree species[1-5]. However, when these methods were adopted to *Eucalyptus camaldulensis* L., brownish product was often obtained. The reason why the products of those method was inferior in quality and/or quantity was supposed that the obstacles were defferent between tree species. Then we made small modification on CTAB method[6] and tried to isolate RNA from differentiating xylem of *E. camaldulensis* L.. CTAB method was originally developed for rapid isolation of DNA from plant tissue[7]. A few investigators adopted it to RNA isolation with some modification and obtain RNA in high yield and in good quality[6,8]. This method would be easy for the most laboratory because of no ultra-centrifugation in its protocol. We described here that RNA

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Abbreviations : CHES = 2-(*N*-cyclohexylamino)ethanesulfonic acid, CTAB = cetyltrimethylammonium bromide, DTT = dithiothreitol, EDTA = ethylenediaminetetraacetic acid, MOPS = 2-(*N*-morpholino)propanesulfonic acid, SDS = sodium dodecyl sulfate, Tris = 2-amino-(hydroxymethyl)-1, 3-propanediol.

was able to be isolated constantly from differentiating xylem of *E. camaldulensis* in high quality and enough quantity.

2. Materials and Methods

2.1 Plant material

Two-years- and three-years-old *E. camaldulensis* (seeds are originated in Petford, Australia) trees which grow in the breeding field of Oji Paper Co. Ltd. (in Kameyama) were used. After main stems were cut off, bark was peeled and the differentiating tissue on the wooden timber was scraped out with a knife blade. The tissue was immediately put into liquid nitrogen (LN_2) and stored at $-80°C$ until use. After a main stem was inclined and fixed for 2 weeks, differentiating xylem of tension wood and opposite wood was also harvested in the same manner.

2.2 Chemicals and experimental materials

Vanadyl Ribonucleoside Complex (VRC) was purchased from Gibco BRL (Gaithersburg, MD, USA), mRNA Purification Kit from Pharmacia Biotech (Uppsala, Sweden), CHES and MOPS from Dojindo Laboratories (Kumamoto, Japan), agarose S (standard type) from Nippon Gene Co. Ltd. (Toyama, Japan), the other chemicals from Wako Pure Chemicals Co. Ltd (Tokyo, Japan). An apparatus for electrophoresis was Mupid 2 (Cosmo Bio Co. Ltd., Tokyo, Japan).

TE contains 10 mM Tris-HCl buffer (pH 8.0) and 1 mM EDTA. Phenol/$CHCl_3$ was made by equal volume of phenol saturated by TE and $CHCl_3$ containing 4% isoamyl alcohol. MOPS buffer contains 20 mM MOPS, 5 mM CH_3COONa, 1 mM EDTA (pH 7.0).

2.3 Isolation of total RNA

The stored tissue (5 g) was ground to fine powder with liquid nitrogen in a mortar. The powdered tissue was more finely ground with homogenizer and glass beads (0.3 mmϕ) the procedure till here was carried out with cooling by liquid nitrogen. Resulting fine powder was suspended in 30 ml of methanol containing 0.1% DTT at room temperature, and centrifuged at 2,200·g for 15 min. Then, supernatant was discarded. This extraction was repeated 3 times, and then pellet was spread on the inner wall of the tube and dried under reduced pressure. To this dried pellet, 25 ml of extraction buffer (100 mM CHES (pH 9.0), 10 mM EDTA, 2% SDS, 0.8% DTT, 10 mM VRC) was added and mixed well. DTT and VRC were mixed to the others just before use. The aliquot was kept at 65°C for 30 min. Then, 7 ml of 5 M NaCl and 2.5 ml of 10% CTAB in 1.4 M NaCl was added and kept at 65°C for more 10 min. Equal volume of $CHCl_3$/isoamyl alcohol (24 : 1) was added and emulsified. It was kept on ice for more than 30 min. It was centrifuged at 2,200·g

for 10 min, and the upper layer was recovered. Isopropanol (0.55 vol) was added to the recovered layer and kept on ice for more than 1 hr. Then, it was centrifuged at 2,200·g for 10 min. Supernatant was discarded and precipitate was dried under reduced pressure. The precipitate was dissolved in 900 μl of sterilized water. To this solution, equal volume of phenol/$CHCl_3$ was added, and after emulsified, it was centrifuged at 18,800·g for 10 min. The upper layer was recovered. This phenol/$CHCl_3$ extract ion was repeated for 3 times. To the final recovered layer, 1/10 vol of 3 M CH_3COONa and 0.6 vol of isopropanol was added and mixed well. It was centrifuged at 18,800·g for 10 min, and precipitate was recovered. The precipitate was washed with 70% ethanol and dried under reduced pressure. After the precipitate was dissolved into sterilized water, 12 M LiCl was added to be 3 M in final concentration and kept on ice for 1 hr. Then it was centrifuged at 18,800·g for 10 min at 4°C and the precipitate was recovered. After washed with 3 M LiCl and 70% ethanol, the precipitate was dissolved in 250 μl of sterilized water. The amount of total RNA was determined by measuring absorbance at 260 nm. The quality of RNA was checked by scanning profile of absorbance between 220–320 nm and electrophoresis on 1.5% agarose S gel containing formaldehyde and MOPS buffer. Poly(A)$^+$ RNA was purified from the RNA by using mRNA Purification Kit as providers recommended. The amount and the quality of poly(A)$^+$ RNA was checked as same as described above.

3. Results and Discussion

The most serious probrem in RNA isolation from differentiating xylem of *Eucalyptus camaldurensis* was brownish products. In most cases, these brown materials are supposed to be tannin or such phenolic compounds. We made two additional modification. One is pre-extraction with methanol containing DTT to remove phenolic compounds, and the other is addition of vanadyl ribonucleosides, which is an inhibitor for RNase, into the extraction buffer. However, vanadyl ribonucleosides would work to remove phenolics also. Methanol pre-extraction decreased the color, but is was not enough. When vanadyl ribonucleosides was omitted from the method, final products colored brownish and the absorbance spectrum showed contamination of phenolics (Fig. 1a). The peak shifted to 270 nm, the spectrum curve had shoulders, and the absorbance of baseline shifted up from zero. It was supposed that vanadyl ribonucleosides would bind to phenolics and protect RNA. An absorbance spectrum of total RNA obtained with these modification was shown in Fig. 1b. The ratio of the optical densities were around 1.9 for 260/280 nm and 2.5 for 260/230 nm. These indicate low amount of contaminating phenolics, proteins and carbohydrates.

The intactness of total RNA and poly(A)$^+$ RNA was checked by electrophoresis in denaturating formaldehyde gel. Two major bands due to rRNA were found. Their front lines of migration were sharp, and indicate low level of digestion. Poly(A)$^+$ RNA obtained

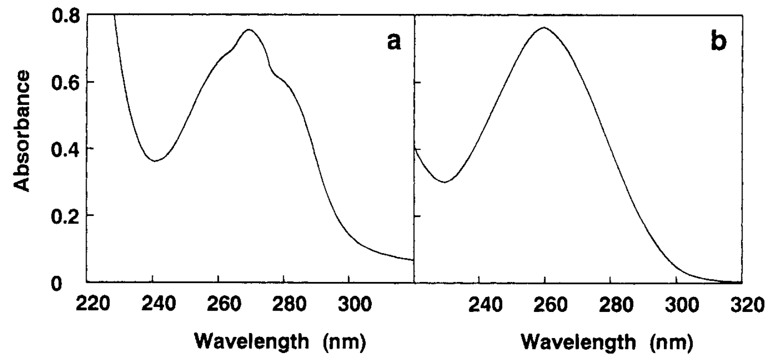

Fig. 1. Absorbance spectra of total RNA obtained from differentiating xylem of normal wood in Eucalyptus camaldulensis L. a: without vanadyl ribonucleosides, b: with vanadyl ribonucleosides.

Table 1. RNA yield from different occasions of differentiating xylem of *Eucalyptus camaldulensis* L

Differentiating xylem of	Yield of RNA (μg RNA/g tissue)	% Poly(A)$^+$ RNA from total RNA
Normal wood	273±27	0.68±0.05
Tension wood	264±32	0.95[a]
Opposite wood	228±48	—[b]

a: Simgle date, b: not attempted (mean±S.E.)

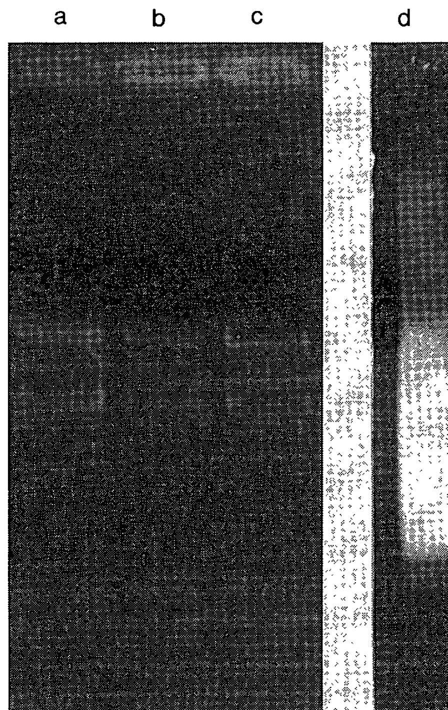

Fig. 2. Electrophoresis of total RNA and poly(A)$^+$ RNA obtained from differentiating xylem of *Eucalyptus camaldulensis* L. Total RNA obtained from: a=normal wood; b=tension wood; c=opposite wood; d=poly(A)$^+$ RNA from tension wood.

from this total RNA was widely spread from top to bottom of the gel, also indicating little digestion during this procedure.

The yield of RNA was ranged from 135–400 μg/g tissue and 273 μg /g in average from differentiating tissue of normal wood (Table 1). It is not excellent but good yield, considering that differentiating xylem is highly vacuolated tissue, in which water occupied higher percentage of tissue wight. Percentage of Poly$(A)^+$ RNA obtained from this total RNA was about 0.7% (Table 1). It shows enough recovery comparing with the other plant tissue[6].

We isolated RNA from differentiating xylem tissues of normal wood, tension wood and opposite wood of *Eucalyptus camaldulensis* by modified CTAB method. Though the composition of each cell wall is different, their yield (Table 1) and quality (Fig. 2) was similar to each other.

These results indicated that total RNA which was obtained by this method had enough quality and quantity for using subsequent several experiments. In fact, we have already constructed cDNA libraries by using Poly$(A)^+$ RNA obtained by this method and some cDNA was cloned from those libraries.

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